

PROTAC-Mediated Degradation of Bruton's Tyrosine Kinase is Inhibited by Covalent Binding

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ABSTRACT: The impact of covalent binding on PROTAC-mediated degradation of BTK was investigated through the preparation of both covalent binding and reversible binding PROTACs derived from the covalent BTK inhibitor ibrutinib. It was determined that a covalent binding PROTAC inhibited BTK degradation despite evidence of target engagement, while BTK degradation was observed with a reversible binding PROTAC. These observations were consistently found when PROTACs were employed that were able to recruit either IAP or cereblon E3 ligases. Proteomics analysis determined that use of a covalently bound PROTAC did not result in the degradation of covalently bound targets, whilst degradation was observed for some reversibly bound targets. This observation highlights the importance of catalysis on successful PROTAC-mediated degradation, and highlights a potential caveat for the use of covalent target binders in PROTAC design.

Protein degrading bifunctional molecules are emerging as a novel drug discovery strategy with the potential to offer pharmacologic control of biology that is not currently achievable with existing small molecule medicines.¹⁻³ Mechanistic hallmarks of proteolysis-targeting chimeras (PROTACs) include high cellular degradation potency (DC₅₀, the concentration at which 50% of substrate is degraded) high target selectivity, and extended pharmacodynamic duration of action that is dependent upon both drug pharmacokinetics and target protein resynthesis rate.^{4,5} The targeted protein degradation paradigm is driven by the ability of PROTACs to catalytically promote the degradation of a desired protein in an event-driven process. This contrasts with occupancy-driven pharmacology that is characteristic of traditional small molecule inhibitors.³

The catalytic mechanism of PROTAC-mediated protein degradation (Figure 1, blue arrows) has been speculated as being fundamental to the high degradation efficiency observed in both *in vitro* and *in vivo* models.¹ This catalytic paradigm creates opportunities to deliver novel medicines with very low doses.³ However, in principle it may also be possible for a covalent binding PROTAC to act as a stoichiometric degrader, resulting in degradation of one protein molecule per PROTAC molecule (Figure 1, green arrows). To explore this hypothesis, the effect of covalent binding on PROTAC-mediated degradation of Bruton's tyrosine kinase (BTK) was tested. BTK was chosen as an exemplar protein target, as it has previously been shown to be degradable *via* the PROTAC approach.^{6,7,8-10} It was envisioned that comparison of structurally similar covalent and non-covalent PROTACs that bind to BTK would elucidate whether the catalytic element of the PROTAC mechanism is important for the efficient degradation of this target. This is important because if stoichiometric PROTACs can be designed for less-tractable targets through the use of covalent target binders to enhance target binding affinity, then this would represent a novel approach for tackling the "undruggable" proteome.^{11,12}

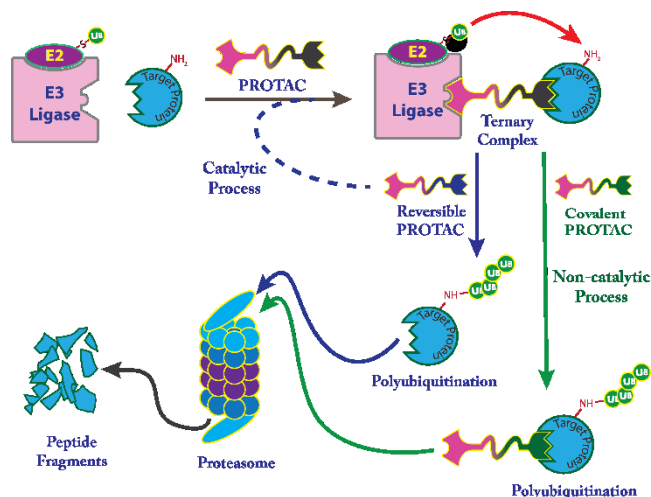


Figure 1. Proposed PROTAC mechanism for catalytic reversible PROTACs (blue) and non-catalytic covalent PROTACs (green).

Covalent-binding PROTACs have previously been shown to be functional target degraders. For example, HaloPROTACs containing a chloroalkane designed to covalently bind to a HaloTag target protein fusion have been used to investigate target degradability.¹³ This method has been shown to induce degradation of specific fusion proteins by recruitment of the E3 ligases VHL¹³ and IAP.¹⁴ Additionally, ERK1/2 was degraded employing a covalent-binding moiety as the targeting element, although covalent binding to ERK1/2 was not confirmed.¹⁵ Although illustrative, these reports do not employ a reversibly binding control compound, therefore the impact of covalent vs. reversible target binding on target degradation cannot be fully characterised.

The current study employs covalent BTK binding moieties chosen such that the covalent-binding element can be removed by formal reduction while still maintaining potent reversible BTK binding and inhibition.¹⁶ This design element allows for a valid comparison of reversible and covalent PROTACs for this target.^{10, 17} The covalent BTK inhibitor ibrutinib **1a** and reversibly binding analogue **1b**¹⁶ were selected for elaboration into PROTACs **2** and **3**, respectively employing a moiety that binds and recruits E3 ligases from the inhibitor of apoptosis proteins (IAP) family.¹⁸ The acrylamide moiety of ibrutinib **1a** was selected as a viable linkage point to the E3 ligase binder due to its solvent exposure in the co-crystal structure of BTK and ibrutinib **1a**,¹⁹ and its use in fluorescent ligands or bioorthogonal probes which maintain BTK binding activity.^{16, 18, 20} This resulted in ibrutinib-based PROTAC **2** and the corresponding reduced acrylamide analogue **3** as a reversible PROTAC control (**Figure 2**). PROTACs **2** and **3** were both observed to have biochemical inhibitory activity against BTK (**Supplementary Figure 1**). PROTAC **2** was found to covalently modify recombinant BTK *in vitro*, as evidenced by intact protein MS, whilst the saturated acrylamide analog **3** was not observed to covalently modify BTK, as expected (**Supplementary Figure 2**).

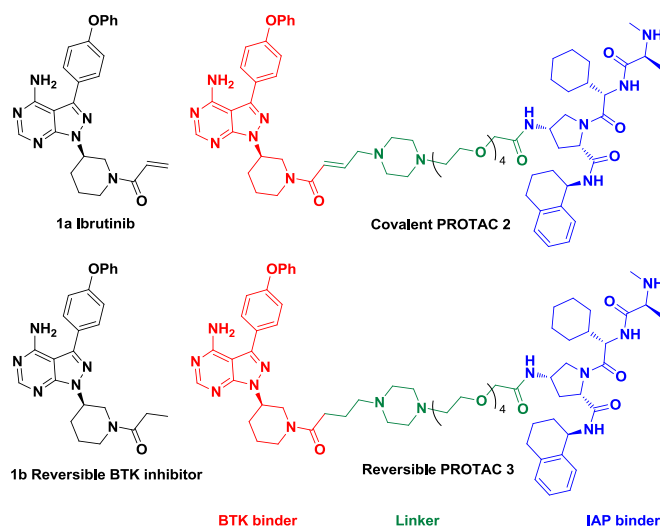


Figure 2: Ibrutinib **1a**, reversible BTK inhibitor **1b**, covalent BTK binding PROTAC **2**, and reversible BTK binding PROTAC **3**.

Both the covalently binding PROTAC **2** and the reversible PROTAC **3** were examined for their ability to cause BTK degradation. THP-1 cells were selected as IAP-mediated degradation of BTK had previously been observed in this cell line.²¹ No BTK degradation was observed upon treatment of THP-1 cells with the covalent PROTAC **2** at increasing concentrations from 1 nM up to 10 μ M for 16 h. (**Figure 3a**). In contrast, THP-1 cell treatment with the reversible PROTAC **3** caused concentration dependent reduction in BTK levels with an approximate DC_{50} of 200 nM (**Figure 3b**). Ibrutinib itself (compound **1a**) was not found to induce BTK degradation (**Supplementary Figure 4**). BTK degradation caused by reversible PROTAC **3** was found to be time dependent in a time course experiment, whilst extended treatment with covalent PROTAC **2** did not result in a significant change to BTK protein levels (**Supplementary Figure 5**).

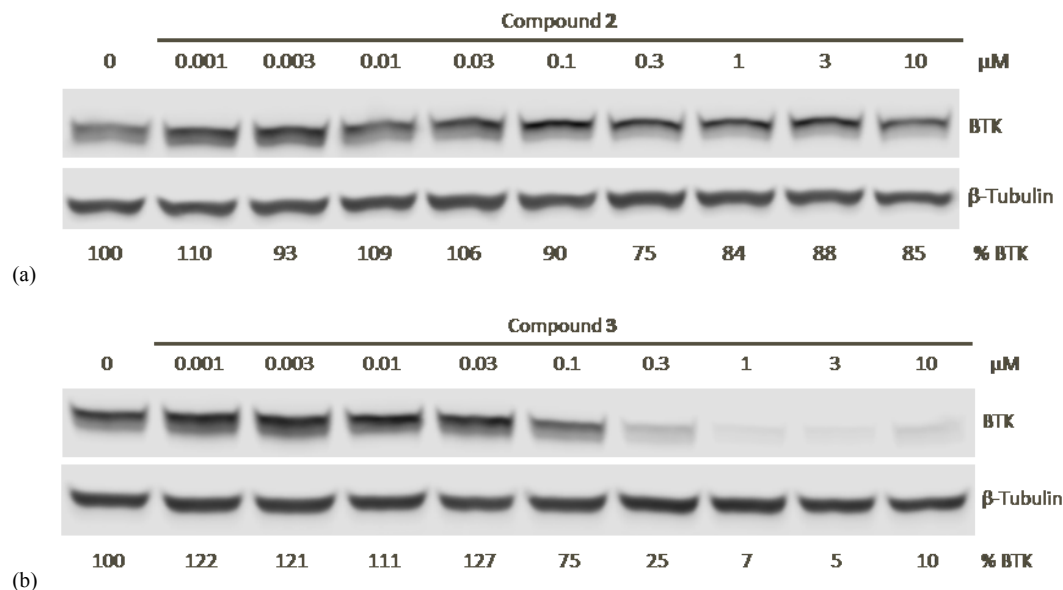


Figure 3: (a) Representative immunoblot of BTK following 16 h incubation with covalent PROTAC **2** at increasing concentrations (b) Representative immunoblot of BTK following 16 h incubation with reversible PROTAC **3** at increasing concentrations. Experiments were conducted in THP-1 cells with β -tubulin as a loading control ($n = 3$, see **Supplementary Figure 3**).

By appropriate selection of cell line, cellular target engagement can be assessed for both the target protein and the E3 ligase for PROTAC compounds **2** and **3**. Kinase domain occupancy of BTK has been shown to correlate with inhibition of BTK autophosphorylation at Y223 following BCR pathway stimulation.¹⁸ Similarly, engagement of the recruited E3 ligase IAP can be assessed, since inhibition of cIAP1 causes its auto-ubiquitination and degradation.²² THP-1 cells are not sensitive to BCR pathway stimulation, therefore this assessment was conducted in Ramos cells.²³ Following stimulation of the BCR pathway with α -IgM in Ramos cells and treatment with covalent PROTAC **2**, concentration dependent reduction of both pBTK Y223 and cIAP1 were observed suggesting engagement of both target protein and E3 ligase at concentrations above 30 nM, however no degradation of BTK was observed (**Figure 4**). Above concentrations of 300 nM, almost complete inhibition of pBTK Y223 was observed, suggesting full occupancy of the kinase domain. At this concentration, it would be expected that BTK degradation would occur in a stoichiometric manner, however there was no observed change to total BTK protein levels, suggesting the lack of BTK degradation is not due to the lack of PROTAC catalysis. For the reversible PROTAC **3**, cIAP1 levels were also reduced at concentrations greater than 30 nM, suggesting effective IAP target engagement and similar cellular penetration as the covalent binding PROTAC **2**. However, BTK autophosphorylation was reduced only at 300 nM suggesting weaker kinase binding as compared to **2**, as expected upon reduction of the acrylamide covalent binding moiety. Despite evidence of reduced BTK target binding, significant BTK degradation was observed upon treatment with the reversible PROTAC **3** at concentrations greater than 300 nM. Ibrutinib **1a** was again found to have no effect on total protein levels in Ramos cells (**Supplementary Figure 4**). The effect of covalent binding on induced BTK degradation was consistent across tested heterobivalent BTK-IAP PROTACs, and was not found to be linker length dependent (**Supplementary Figure 7**). Degradation of BTK was confirmed to be a result of IAP ligase family recruitment, since diastereomers of PROTACs **2** and **3** employing the inactive enantiomer of the parent IAP inhibitor did not degrade BTK at all concentrations tested (**Supplementary Figure 8**).

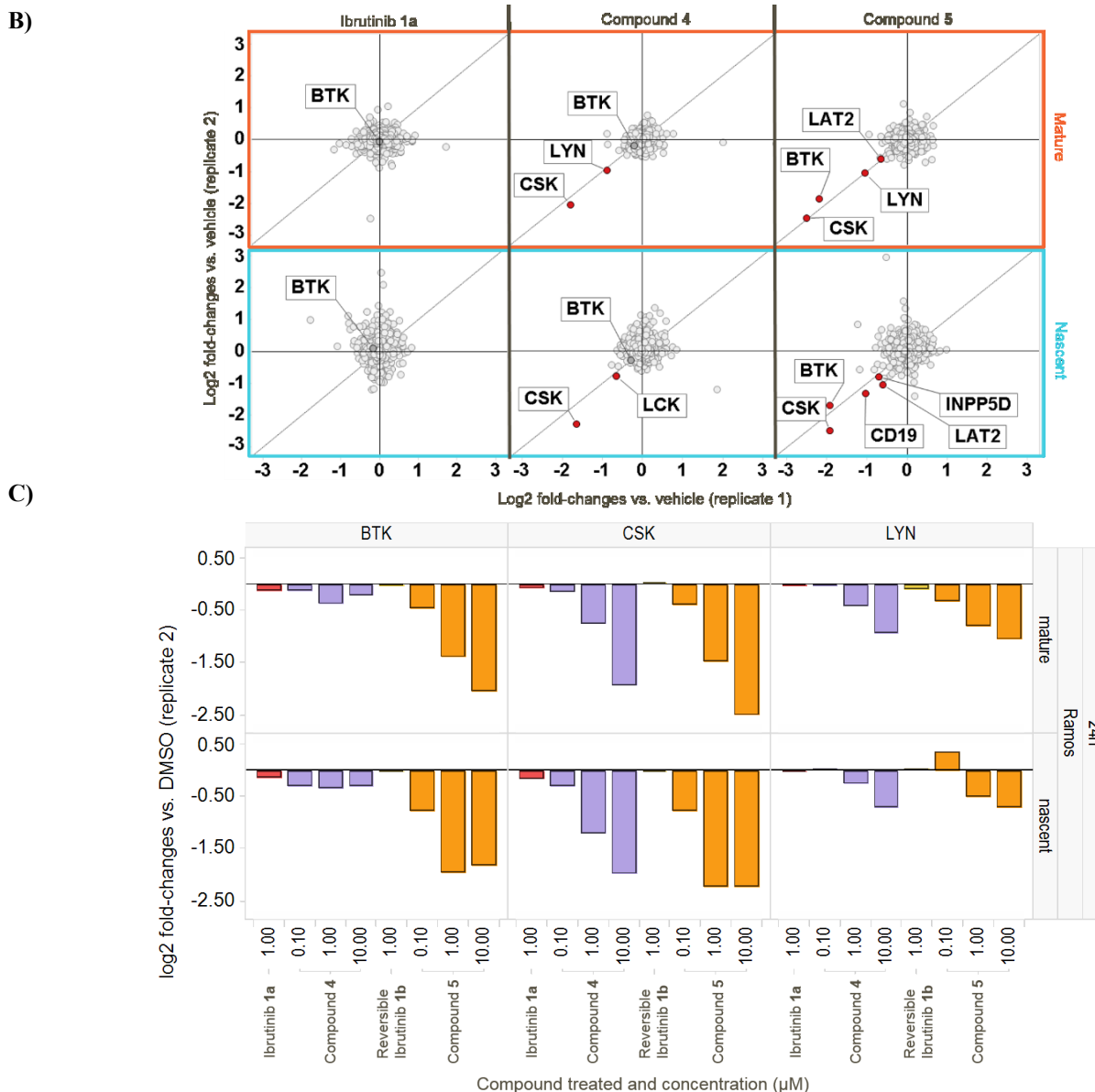


Figure 5: **A)** Cereblon PROTACs targeting BTK (PROTACs **4** and **5**). **B)** Multiplexed proteome dynamics profiling of Ramos cells upon PROTAC treatment. Ramos cells were incubated with either ibrutinib **1a** (1 μ M), covalent PROTAC **4** (10 μ M) or reversible PROTAC **5** (10 μ M) for 24 h. Discrimination of mature and nascent proteins was enabled by a pulse SILAC approach. The graphs show fold change values (\log_2 scale) of abundance compared to the vehicle control of replicate 2 over replicate 1 for each condition. Effects on mature proteins and nascent proteins are shown in the upper panels framed in orange and in the lower panels framed in blue, respectively. Proteins significantly regulated in at least one condition (fold change $\geq 33\%$ and p value ≤ 0.05) are marked in red. **C)** Multiplexed proteome dynamics profiling of Ramos cells upon compound treatment, showing fold change values (\log_2 scale) for representative replicate 2 for BTK, CSK and LYN. Cellular treatment with ibrutinib **1a** or the reversible ibrutinib analogue **1b** (1 μ M), compound **4** (0.1, 1 and 10 μ M), and compound **5** (0.1, 1 and 10 μ M).

The lack of PROTAC-mediated degradation of BTK observed with ibrutinib-derived covalent binding PROTACs **2** and **4** highlights a potential caveat for covalent-based approaches for the induction of targeted protein degradation, as our experiments show that irreversible target binding blocks IAP or cereblon-mediated degradation of this target. Potential explanations for this unusual effect may be that the covalently bound ligand blocks ubiquitin transfer onto a surface lysine required for degradation, or the covalent protein modification blocks the presentation of the intrinsically disordered region required for recognition by the proteasome.²⁹ In contrast, PROTACs that reversibly bind to BTK can induce the degradation of BTK, but may not induce the degradation of other kinases bound by the molecule, as suggested by the results observed with PROTAC **5**, and separately by Crews and Gray.^{6, 7} This work suggests that despite literature evidence indicating that PROTAC molecules can successfully induce the degradation of covalently bound protein targets,^{13, 14} different factors likely play a significant role in the degradation of covalently bound PROTAC targets as compared to reversibly bound PROTAC targets.

ASSOCIATED CONTENT

Supporting Information

Supporting information, including supplementary figures, materials and methods, and compound characterisation is available free of charge via the internet at <http://pubs.acs.org>

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Author Contributions

±These authors contributed equally. C.P.T., H.L. synthesised compounds and performed intact protein MS. L.D. synthesised compounds and performed proteomics study. L.D., M.B. conceptualized proteomics study and analysed results. Z.I.B., S.E.H. prepared immunoblots. H.D. managed biological outsourcing. M.M., I.E.D.S., W.J.K., G.A.B., J.D.H. provided supervisory support. C.P.T., H. L. prepared the manuscript.

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ABBREVIATIONS

PROTAC, Proteolysis Targeting Chimera, IAP, inhibitor of apoptosis protein, XIAP, X-linked inhibitor of apoptosis protein, cIAP1, cellular inhibitor of apoptosis protein-1, BTK, Bruton's Tyrosine Kinase, VHL, Von Hippel-Lindau, ERK1/2, Extracellular signal-regulated kinases, BCR, breakpoint cluster region protein, CSK, C-terminal Src kinase, LAT2, Linker for Activation of T-cell Family Member2, CD19, Cluster of Differentiation 19, INPP5D, Inositol polyphosphate-5-phosphatase.

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